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Systematic Chemoenzymatic Synthesis of *O*-Sulfated Sialyl Lewis x Antigens.

### Permalink

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### Journal

Chemical science, 7(4)

### ISSN

2041-6520

### Authors

Santra, Abhishek  
Yu, Hai  
Tasnima, Nova  
et al.

### Publication Date

2016-04-01

### DOI

10.1039/c5sc04104j

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click for updatesCite this: *Chem. Sci.*, 2016, 7, 2827Systematic chemoenzymatic synthesis of *O*-sulfated sialyl Lewis x antigens†Abhishek Santra,<sup>a</sup> Hai Yu,<sup>a</sup> Nova Tasnima,<sup>a</sup> Musleh M. Muthana,<sup>‡a</sup> Yanhong Li,<sup>a</sup> Jie Zeng,<sup>ab</sup> Nicholas J. Kenyon,<sup>c</sup> Angelique Y. Louie<sup>d</sup> and Xi Chen<sup>\*a</sup>

*O*-Sulfated sialyl Lewis x antigens play important roles in nature. However, due to their structural complexity, they are not readily accessible by either chemical or enzymatic synthetic processes. Taking advantage of a bacterial sialyltransferase mutant that can catalyze the transfer of different sialic acid forms from the corresponding sugar nucleotide donors to Lewis x antigens, which are fucosylated glycans, as well as an efficient one-pot multienzyme (OPME) sialylation system, *O*-sulfated sialyl Lewis x antigens containing different sialic acid forms and *O*-sulfation at different locations were systematically synthesized by chemoenzymatic methods.

Received 29th October 2015  
Accepted 14th December 2015

DOI: 10.1039/c5sc04104j

www.rsc.org/chemicalscience

## Introduction

*O*-Sulfated sialyl Lewis x structures play important roles in immune regulation, inflammation, and cancer metastasis.<sup>1</sup> For example, 6-*O*-sulfo-sialyl Lewis x [6-*O*-sulfo-sLe<sup>x</sup> (1), Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc6SβOR] with an *O*-sulfate group at the carbon-6 of the *N*-acetylglucosamine (GlcNAc) residue (Fig. 1) is a well known ligand for L-selectin, a C-type (Ca<sup>2+</sup>-dependent) carbohydrate-binding protein (lectin) expressed broadly in most leukocytes in the blood.<sup>1,2</sup> The interaction of 6-*O*-sulfo-sLe<sup>x</sup> (1) and L-selectin plays a critical role in lymphocyte homing to the peripheral lymph nodes<sup>2</sup> and in chronic inflammation.<sup>3</sup> It has also been shown that human sialic acid-binding immunoglobulin-like lectin<sup>4</sup> Siglec-9 binds strongly<sup>5,6</sup> to 6-*O*-sulfo-sLe<sup>x</sup>, but the biological importance of this interaction is less well understood.

On the other hand, 6'-*O*-sulfo-sialyl Lewis x [6'-*O*-sulfo-sLe<sup>x</sup> (2), Neu5Acα2-3Gal6Sβ1-4(Fucα1-3)GlcNAcβOR] with an *O*-sulfate group at the carbon-6 of the galactose (Gal) residue (Fig. 1),<sup>7</sup> in addition to 6'-*O*-sulfo-sialyl-*N*-acetylglucosamine (6'-*O*-sulfo-sLacNAc, Neu5Acα2-3Gal6Sβ1-4GlcNAcβOR),<sup>8</sup> was

shown by glycan microarray studies to be a preferred glycan ligand for Siglec-8 and for its paralog mouse Siglec-F.<sup>9</sup> Siglec-8 is expressed in human allergic inflammatory cells including eosinophils, mast cells, and basophils.<sup>5,10</sup> Reducing the number of eosinophils, such as by soluble 6'-*O*-sulfo-sLe<sup>x</sup> synthetic polymer induced apoptosis,<sup>11</sup> has been suggested as an approach for asthma therapies.<sup>12</sup> Furthermore, 6'-*O*-sulfo-sLe<sup>x</sup> (2), in addition to 6'-*O*-sulfo-sLacNAc and 6'-*O*-sulfo-sialyl-lacto-*N*-neotetraose (6'-*O*-sulfo-sLNT, Neu5Acα2-3Gal6Sβ1-4GlcNAcβ1-3Galβ1-4GlcβOR), was shown to bind to langerin,<sup>13</sup> a C-type (Ca<sup>2+</sup>-dependent) lectin specific to Langerhans cells (immature antigen-presenting specific T cell immunity initiating dendritic cells of epidermis and mucosal tissues).<sup>14</sup>

Although less efficient than Neu5Acα2-8Neu5Acα2-3LacNAc, both 6-*O*-sulfo-sLe<sup>x</sup> (1) and 6'-*O*-sulfo-sLe<sup>x</sup> (2) bound moderately

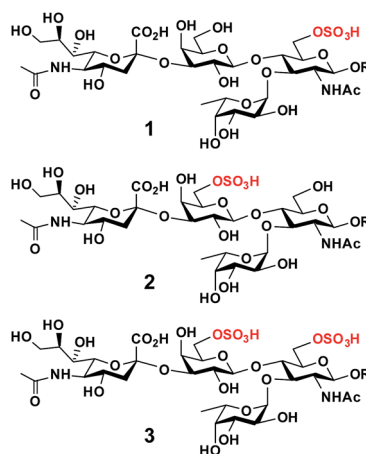


Fig. 1 Structures of *O*-sulfated sialyl Lewis x including 6-*O*-sulfo-sLe<sup>x</sup> (1), 6'-*O*-sulfo-sLe<sup>x</sup> (2), and 6', 6-di-*O*-sulfo-sLe<sup>x</sup> (3).

<sup>a</sup>Department of Chemistry, University of California, Davis One Shields Avenue, Davis, CA 95616, USA. E-mail: xiuchen@ucdavis.edu; Fax: +1-530-752-8995; Tel: +1-530-754-6037

<sup>b</sup>School of Food Science, Henan Institute of Science and Technology, Xinxiang, 453003, China

<sup>c</sup>Division of Pulmonary, Critical Care and Sleep Medicine, Department of Internal Medicine, University of California, Davis, CA 95616, USA

<sup>d</sup>Department of Biomedical Engineering, University of California, Davis, CA 95616, USA

† Electronic supplementary information (ESI) available: Materials, experimental details of the synthesis, analytical data of 4–10, 1a–3a, and 1b–3b, and NMR spectra of synthesized compounds. See DOI: 10.1039/c5sc04104j

‡ Current address: Children's National Medical Center, Washington DC, 20010, USA.

to human Siglec-7.<sup>5</sup> Both are present in glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1), an L-selectin ligand,<sup>15</sup> with 6'-*O*-sulfo-sLe<sup>x</sup> (2) as the major sulfated form.<sup>16–18</sup> Gal-6-*O*-sulfotransferase and GlcNAc-6-*O*-sulfotransferase have been found to synergistically produce L-selectin ligands. This indicates either the potential synergistic involvement of both 6-*O*-sulfo-sLe<sup>x</sup> (1) and 6'-*O*-sulfo-sLe<sup>x</sup> (2) or the involvement of 6',6-di-*O*-sulfo-sLe<sup>x</sup> (3) (Fig. 1) with *O*-sulfate groups at both the Gal and GlcNAc residues of sLe<sup>x</sup> in L-selectin-binding.<sup>19</sup> Human Siglec-7 and -8 have also been shown to bind more strongly to 6',6-di-*O*-sulfo-sLe<sup>x</sup> (3) than their mono-*O*-sulfated derivatives (1 and 2), while mouse Siglec-F has been shown to bind with similar strength to 6',6-di-*O*-sulfo-sLe<sup>x</sup> (3) and 6'-*O*-sulfo-sLe<sup>x</sup> (2).<sup>6</sup>

The biological importance of *O*-sulfated sLe<sup>x</sup> structures makes them attractive synthetic targets. However, the structures of these compounds are relatively complex and include synthetically challenging  $\alpha$ 2-3-linked sialic acid, which suffers from low stereoselectivity and a high 2,3-elimination rate in chemical synthesis,<sup>20–22</sup> as well as the acid labile *O*-sulfate group.<sup>23,24</sup> Chemically<sup>20,25,26</sup> or chemoenzymatically<sup>27</sup> synthesized Neu5Ac $\alpha$ 2-3Gal building blocks have been used as effective synthons for constructing more complex sialosides including sLe<sup>x</sup> and 6-*O*-sulfo-sLe<sup>x</sup> (1).<sup>20</sup> Several examples of the chemical<sup>22,28</sup> or chemoenzymatic<sup>29</sup> synthesis of 6-*O*-sulfo-sLe<sup>x</sup> (1) as well as the chemical synthesis of 6'-*O*-sulfo-sLe<sup>x</sup> (2)<sup>22,30,31</sup> and 6',6-di-*O*-sulfo-sLe<sup>x</sup> (3)<sup>32</sup> have been reported. All these examples are, however, limited to compounds with the most abundant sialic acid form, *N*-acetylneuraminic acid (Neu5Ac). Despite the presence of more than 50 different sialic acid forms

identified in nature,<sup>33,34</sup> *O*-sulfated sLe<sup>x</sup> containing a sialic acid form other than Neu5Ac has not been synthesized.

We report here the development of efficient chemoenzymatic methods for the systematic synthesis of *O*-sulfated sLe<sup>x</sup> containing different sialic acid forms. The methods are demonstrated for representative examples of 6'-*O*-sulfo-sLe<sup>x</sup> (1), 6-*O*-sulfo-sLe<sup>x</sup> (2) and/or 6',6-di-*O*-sulfo-sLe<sup>x</sup> (3) containing the most abundant Neu5Ac form and *N*-glycolylneuraminic acid (Neu5Gc), a sialic acid form commonly found in mammals other than humans, but which can be incorporated into the human glycome from dietary sources.<sup>35</sup>

One efficient approach for the synthesis of *O*-sulfated sLe<sup>x</sup> with different sialic acid forms would be by direct sialylation of *O*-sulfated Le<sup>x</sup> using one-pot multienzyme (OPME) sialylation systems<sup>36</sup> containing an  $\alpha$ 2-3-sialyltransferase and a CMP-sialic acid synthetase (CSS),<sup>37</sup> with or without a sialic acid aldolase.<sup>38</sup> Such an approach has been successfully demonstrated for direct sialylation of non-sulfated Le<sup>x</sup> for the synthesis of sLe<sup>x</sup> containing a diverse array of naturally occurring and non-natural sialic acid forms, using OPME systems containing a recombinant viral  $\alpha$ 2-3-sialyltransferase vST3Gal-I<sup>39</sup> or a bacterial multifunctional sialyltransferase mutant, *Pasteurella*

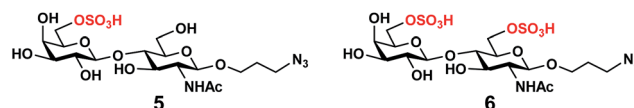
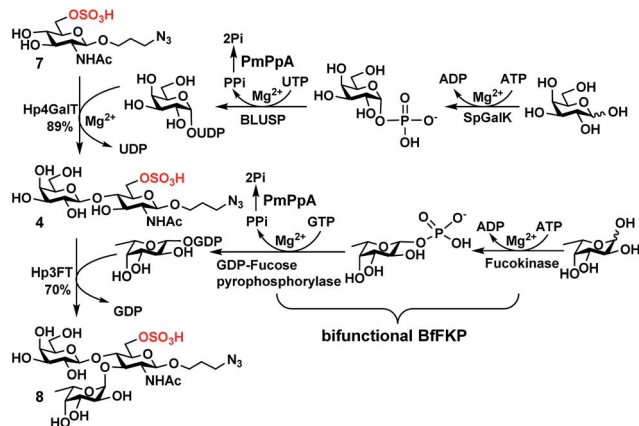
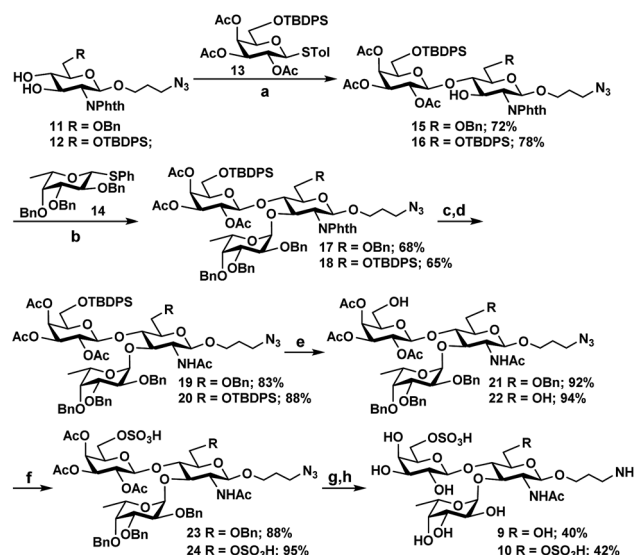


Fig. 2 Structures of chemically synthesized 6'-*O*-sulfo-LacNAc $\beta$ ProN<sub>3</sub> (5) and 6',6-di-*O*-sulfo-LacNAc $\beta$ ProN<sub>3</sub> (6).

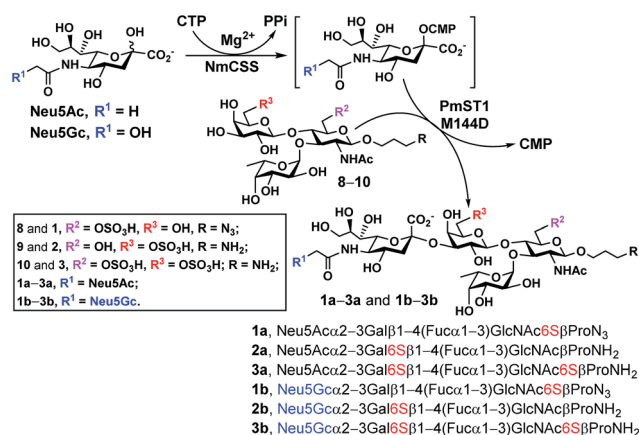


Scheme 1 Sequential OPME synthesis of 6-*O*-sulfo-Le<sup>x</sup> $\beta$ ProN<sub>3</sub> (8) from 6-*O*-sulfo-GlcNAc $\beta$ ProN<sub>3</sub> (7) using an OPME  $\beta$ 1-4-galactosyl activation and transfer system for the formation of 6-*O*-sulfo-LacNAc $\beta$ ProN<sub>3</sub> (4) followed by an OPME  $\alpha$ 1-3-fucosyl activation and transfer system for the formation of 6-*O*-sulfo-Le<sup>x</sup> $\beta$ ProN<sub>3</sub> (8). Enzymes and abbreviations: SpGalK, *Streptococcus pneumoniae* TIGR4 galactokinase;<sup>44</sup> BLUSP, *Bifidobacterium longum* UDP-sugar pyrophosphorylase;<sup>45</sup> PmPpA, *Pasteurella multocida* inorganic pyrophosphorylase;<sup>46</sup> Hp4GalT, *Helicobacter pylori*  $\beta$ 1-4-galactosyltransferase;<sup>43</sup> BFFKP, *Bacteroides fragilis* bifunctional L-fucokinase/GDP-fucose pyrophosphorylase;<sup>42</sup> and Hp3FT, *Helicobacter pylori*  $\alpha$ 1-3-fucosyltransferase.<sup>39,41</sup>



Scheme 2 Chemical synthesis of 6'-*O*-sulfo-Le<sup>x</sup> $\beta$ ProNH<sub>2</sub> (9) and 6',6-di-*O*-sulfo-Le<sup>x</sup> $\beta$ ProNH<sub>2</sub> (10). Reagents and conditions: (a) *N*-iodosuccinimide (NIS), TMSOTf, MS 4 Å, CH<sub>2</sub>Cl<sub>2</sub>, -40 °C, 30 min; (b) *N*-iodosuccinimide (NIS), TMSOTf, MS 4 Å, CH<sub>2</sub>Cl<sub>2</sub>-Et<sub>2</sub>O (1 : 1), -18 °C, 45 min; (c) H<sub>2</sub>N(CH<sub>2</sub>)<sub>2</sub>NH<sub>2</sub>, *n*-BuOH, 90 °C, 8 h; (d) pyridine, Ac<sub>2</sub>O, r.t., 10 h; (e) HF pyridine, 0 °C to r.t., overnight; (f) SO<sub>3</sub> pyridine, pyridine, 0 °C to r.t.; (g) 0.1 M NaOMe, MeOH, r.t., 3 h; (h) Pd(OH)<sub>2</sub>/C, H<sub>2</sub>, CH<sub>3</sub>OH, 48 h.





**Scheme 3** PmST1 M144D-mediated one-pot two-enzyme (OP2E) sialylation of *O*-sulfo analogues of Lewis<sup>x</sup>. Yields obtained for *O*-sulfated sLe<sup>x</sup> tetrasaccharides: 1a, 85%; 1b, 47%; 2a, 82%; 2b, 60%; 3a, 64%; 3b, 38%. Enzymes and abbreviations: NmCSS, *Neisseria meningitidis* CMP-sialic acid;<sup>37</sup> PmST1 M144D, *Pasteurella multocida*  $\alpha$ 2-3-sialyltransferase 1 (PmST1) M144D mutant.<sup>40</sup>

*multocida*  $\alpha$ 2-3-sialyltransferase 1 (PmST1) M144D.<sup>40</sup> The latter, with a high expression level (98 mg L<sup>-1</sup> culture, >1000-fold higher than that of vST3Gal-I) and high promiscuity in tolerating different modifications on the sialic acid in the substrates, is a superior choice for the synthesis.<sup>40</sup> However, it is not clear whether *O*-sulfated Le<sup>x</sup> structures could be used with PmST1 M144D as suitable acceptors in the OPME sialylation process to produce the desired *O*-sulfated sLe<sup>x</sup> with different sialic acid forms.

## Results and discussion

### Synthesis of *O*-sulfated disaccharides and *O*-sulfated Le<sup>x</sup>

In order to obtain *O*-sulfated Le<sup>x</sup> as potential acceptor substrates for PmST1 M144D, enzyme-catalyzed  $\alpha$ 1-3-fucosylation of the corresponding *O*-sulfated disaccharides was tested as a potential strategy. A one-pot three-enzyme (OP3E)  $\alpha$ 1-3-fucosylation system (Scheme 1)<sup>39,41</sup> containing *Bacteroides fragilis* bifunctional  $\alpha$ -fucokinase/GDP-fucose pyrophosphorylase (BfFKP),<sup>42</sup> *Pasteurella multocida* inorganic pyrophosphorylase (PmPpA),<sup>43</sup> and *Helicobacter pylori*  $\alpha$ 1-3-fucosyltransferase (Hp1-3FT $\Delta$ 66 or Hp3FT) was used for this purpose. The *O*-sulfated disaccharides tested were 6-*O*-sulfo-LacNAc $\beta$ ProN<sub>3</sub> (4) (Scheme 1), 6'-*O*-sulfo-LacNAc $\beta$ ProN<sub>3</sub> (5), and 6,6'-di-*O*-sulfo-LacNAc $\beta$ ProN<sub>3</sub> (6) (Fig. 2). LacNAc $\beta$ ProN<sub>3</sub> (ref. 43) without any *O*-sulfate groups was used as a positive control.

6-*O*-Sulfo-LacNAc $\beta$ ProN<sub>3</sub> (4) was synthesized from 6-*O*-sulfo-GlcNAc $\beta$ ProN<sub>3</sub> (7)<sup>43</sup> using an improved OPME galactosyl activation and transfer system (Scheme 1) containing *Streptococcus pneumoniae* TIGR4 galactokinase (SpGalK),<sup>44</sup> *Bifidobacterium longum* UDP-sugar pyrophosphorylase (BLUSP),<sup>45</sup> PmPpA, and a *Helicobacter pylori*  $\beta$ 1-4-galactosyltransferase (Hp1-4GalT or Hp4GalT).<sup>43</sup> The EcGalK, BLUSP, and PmPpA allowed *in situ* formation of the donor substrate of Hp4GalT, uridine 5'-diphosphate-galactose (UDP-Gal), from monosaccharide

galactose (Gal).<sup>45</sup> It was previously shown that Hp4GalT, but not *Neisseria meningitidis*  $\beta$ 1-4-galactosyltransferase (NmLgtB), was able to use 6-*O*-sulfated GlcNAc and derivatives as acceptor substrates for the synthesis of  $\beta$ 1-4-linked galactosides.<sup>43</sup> The activity of Hp4GalT in synthesizing 6-*O*-sulfo-LacNAc $\beta$ ProN<sub>3</sub> (4) was confirmed again here using the improved OPME approach.<sup>45,46</sup> An excellent 89% yield was obtained, comparing favourably to the previous Hp4GalT-dependent OPME  $\beta$ 1-4-galactosylation approach (70% yield) which used *Escherichia coli* K-12 glucose-1-P uridylyltransferase (EcGalU), *Escherichia coli* UDP-galactose-4-epimerase (EcGalE), and PmPpA to produce UDP-Gal *in situ* from glucose-1-phosphate.<sup>43</sup> 6'-*O*-Sulfo-LacNAc $\beta$ ProN<sub>3</sub> (5) and 6,6'-di-*O*-sulfo-LacNAc $\beta$ ProN<sub>3</sub> (6) (Fig. 2) were chemically synthesized (see ESI†).

Among the three *O*-sulfated disaccharides tested, only 6-*O*-sulfo-LacNAc $\beta$ ProN<sub>3</sub> (4) was a suitable acceptor for Hp3FT to produce the desired 6-*O*-sulfo-Le<sup>x</sup> $\beta$ ProN<sub>3</sub> (8). In contrast, 6'-*O*-sulfo-LacNAc $\beta$ ProN<sub>3</sub> (5) and 6,6'-di-*O*-sulfo-LacNAc $\beta$ ProN<sub>3</sub> (6) were not used efficiently by Hp3FT for the synthesis of the corresponding *O*-sulfated Le<sup>x</sup> derivatives. With the positive outcome in small scale reactions for fucosylation of 6-*O*-sulfo-LacNAc $\beta$ ProN<sub>3</sub> (4), the preparative-scale synthesis of 6-*O*-sulfo-Le<sup>x</sup> $\beta$ ProN<sub>3</sub> (8) was carried out using the OP3E  $\alpha$ 1-3-fucosyl activation and transfer system (Scheme 1). A yield of 70% was obtained. The combined sequential OPME  $\beta$ 1-4-galactosylation and OPME  $\alpha$ 1-3-fucosylation (Scheme 1) was an effective approach for obtaining 6-*O*-sulfo-Le<sup>x</sup> $\beta$ ProN<sub>3</sub> (8) from a simple monosaccharide derivative 6-*O*-sulfo-GlcNAc $\beta$ ProN<sub>3</sub> (7) in an overall yield of 62%.

As Hp3FT was not able to use 6'-*O*-sulfo-LacNAc $\beta$ ProN<sub>3</sub> (5) or 6,6'-di-*O*-sulfo-LacNAc $\beta$ ProN<sub>3</sub> (6) efficiently as acceptors for fucosylation to obtain the desired Le<sup>x</sup> trisaccharides, the target trisaccharides 6'-*O*-sulfo-Le<sup>x</sup> $\beta$ ProNH<sub>2</sub> (9) and 6,6'-di-*O*-sulfo-Le<sup>x</sup> $\beta$ ProNH<sub>2</sub> (10) were chemically synthesized (Scheme 2) from monosaccharide synthons 11, 12,<sup>27</sup> 13, and 14.<sup>27</sup> Notable features of the synthetic strategy include: (a) application of an efficient general protection strategy<sup>47</sup> for the synthesis of the two trisaccharides (*i.e.* similar protecting groups were used in the syntheses and the same reagents were used for their removal); (b) use of similar thioglycoside derivatives as glycosyl donors in all glycosylations; (c) high regio- and stereoselectivity in product formation; (d) one step removal of benzyl ethers and reduction of the azido group using 20% Pd(OH)<sub>2</sub>/C (Pearlman's catalyst) and H<sub>2</sub>.<sup>48</sup> More specifically, for the synthesis of 9 and 10, two *N*-phthalimide glucosamine derivatives 11 and 12 selectively protected at C6 with benzyl and *tert*-butyldiphenylsilyl ether (TBDPS), respectively, were coupled stereoselectively with thioglycoside donor 13, which was selectively protected with TBDPS at C6, in the presence of *N*-iodosuccinimide (NIS) and trimethylsilyl trifluoromethanesulfonate (TMSOTf)<sup>49</sup> in dichloromethane. Disaccharide derivatives 15 and 16 were obtained in 72% and 78% yields, respectively. The bulky *N*-phthalimido protecting group in acceptors 11 and 12 provides steric hindrance to the neighboring C-3 hydroxyl group and decreases the reactivity of the C-3 hydroxyl group. Therefore, glycosylation occurs regioselectively at the C-4 hydroxyl group.<sup>27</sup> Initial attempts to glycosylate acceptors 15 and 16 in





dichloromethane with 1.2 equivalents of thiophenyl fucoside **14** produced trisaccharides in alpha and beta mixtures. In contrast, stereospecific formation of trisaccharides was achieved when a mixed solvent of diethylether and dichloromethane (1 : 1)<sup>50,51</sup> was employed. The reaction of acceptors **15** and **16** with 1.2 equivalents of fucosyl donor **14** produced compounds **17** and **18** in 68% and 65% yields, respectively. Compounds **17** and **18** were then subjected to a series of synthetic transformations: (a) conversion of the *N*-phthaloyl group to an acetamido group by removing the phthaloyl group using ethylenediamine, followed by *N*- and *O*-acetylation using acetic anhydride and pyridine; (b) HF-pyridine-mediated selective removal of the TBDPS group;<sup>52</sup> (c) *O*-sulfation of the primary hydroxyl group by SO<sub>3</sub> pyridine complex;<sup>52,53</sup> (d) deacetylation by NaOMe in MeOH;<sup>54</sup> and (e) hydrogenation using Pd(OH)<sub>2</sub>/C and H<sub>2</sub> (ref. 55) to obtain the desired 6'-*O*-sulfo-Le<sup>x</sup>βProNH<sub>2</sub> (**9**) and 6,6'-di-*O*-sulfo-Le<sup>x</sup>βProNH<sub>2</sub> (**10**).

### Enzymatic synthesis of *O*-sulfated sLe<sup>x</sup>

With chemoenzymatically synthesized 6-*O*-sulfo-Le<sup>x</sup>βProN<sub>3</sub> (**8**) as well as chemically synthesized 6'-*O*-sulfo-Le<sup>x</sup>βProNH<sub>2</sub> (**9**) and 6,6'-di-*O*-sulfo-Le<sup>x</sup>βProNH<sub>2</sub> (**10**) in hand, a one-pot two-enzyme (OP2E) sialylation system (Scheme 3) was used to test the tolerance of PmST1 M144D<sup>40</sup> for using these *O*-sulfated Le<sup>x</sup> compounds as potential acceptor substrates. PmST1 M144D was previously engineered by protein crystal structure-assisted design. It has 20-fold reduced CMP-sialic acid (donor) hydrolysis activity and significantly (5588-fold) decreased α2-3-sialidase activity compared to the wild-type enzyme. It was used efficiently in a one-pot three-enzyme (OP3E) sialylation system for the synthesis of non-sulfated sLe<sup>x</sup> tetrasaccharides containing diverse sialic acid forms from Le<sup>x</sup>.<sup>40</sup> To our delight, PmST1 M144D also tolerated *O*-sulfated Le<sup>x</sup> containing *O*-sulfate at C-6, C-6', or both. In addition to *N*-acetylneuraminic acid (Neu5Ac), *N*-acetylneuraminic acid (Neu5Gc) was also successfully introduced to compounds **8–10**. *O*-Sulfated sLe<sup>x</sup> tetrasaccharides 6-*O*-sulfo-Neu5Acα2-3Le<sup>x</sup>βProN<sub>3</sub> (**1a**, 80 mg, 85%), 6-*O*-sulfo-Neu5Gcα2-3Le<sup>x</sup>βProN<sub>3</sub> (**1b**, 22 mg, 47%), 6'-*O*-sulfo-Neu5Acα2-3Le<sup>x</sup>βProNH<sub>2</sub> (**2a**, 75 mg, 82%), 6'-*O*-sulfo-Neu5Acα2-3Le<sup>x</sup>βProNH<sub>2</sub> (**2b**, 45 mg, 60%), 6,6'-di-*O*-sulfo-Neu5Acα2-3Le<sup>x</sup>βProNH<sub>2</sub> (**3a**, 42 mg, 64%), and 6,6'-di-*O*-sulfo-Neu5Gcα2-3Le<sup>x</sup>βProNH<sub>2</sub> (**3b**, 40 mg, 38%) were successfully obtained using this highly efficient one-pot two-enzyme system containing *Neisseria meningitidis* CMP-sialic acid (NmCSS)<sup>37</sup> and PmST1 M144D<sup>40</sup> from the corresponding acceptors **8–10** and Neu5Ac or Neu5Gc, respectively. In general, Neu5Gc was used less efficiently by the OPME sialylation system, leading to lower yields for **1b–3b** (38–60%) compared to their Neu5Ac-counterparts **1a–3a** (64–85%). *O*-Sulfated sLe<sup>x</sup> glycans with a propyl amine aglycone (compounds **2a**, **3a**, **2b** and **3b**) were found to be more challenging for column purification compared to the ones with a propyl azide aglycone (compounds **1a** and **1b**). When a desired sialic acid is readily available such as in the case presented here, a one-pot two-enzyme (OP2E) system is sufficient. When only the 6-carbon precursors of the desired sialic acid forms are available, the one-pot three-enzyme (OP3E)

sialylation system including an aldolase in addition to NmCSS and PmST1 M144D<sup>40</sup> should be used.

## Conclusions

In conclusion, we have successfully developed an efficient chemoenzymatic method for the systematic synthesis of synthetically challenging *O*-sulfated sLe<sup>x</sup> (**1a–3a** and **1b–3b**) containing different sialic acid forms (Neu5Ac or Neu5Gc) by direct sialylation of the corresponding *O*-sulfated Le<sup>x</sup> structures **8–10** using an efficient one-pot two-enzyme (OP2E) system containing NmCSS and PmST1 M144D. The method can be extended to the synthesis of *O*-sulfated sLe<sup>x</sup> structures containing other sialic acid forms. We have also shown here that a relatively complex trisaccharide 6-*O*-sulfo-Le<sup>x</sup>βProN<sub>3</sub> (**8**) can be efficiently produced from a simple monosaccharide derivative 6-*O*-sulfo-GlcNAcβProN<sub>3</sub> (**7**) by a sequential OPME β1-4-galactosylation and OPME α1-3-fucosylation process. PmST1 M144D has been demonstrated to be a powerful catalyst not only for synthesizing non-sulfated sLe<sup>x</sup> structures as shown previously,<sup>40</sup> but also for producing biologically important but difficult-to-obtain *O*-sulfated sLe<sup>x</sup>.

## Acknowledgements

This work was supported by NIH grants R01GM094523 (to X. C.) and R21AI097354 (to A. Y. L.) as well as NSF grant CHE-1300449 (to X. C.). M. M. M. acknowledges UC-Davis and USDE for a GAANN Fellowship (P200A120187). A Bruker Avance-800 NMR spectrometer was funded by NSF grant DBIO-722538.

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